Identification of genes involved in PHB biosynthesis in bacterial isolates and characterization of high PHB producing mutant

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Introduction:

Synthetic plastics have made entry in every sphere of human life from water bottles to prosthetics, plastics can be seen everywhere. Excessive use of synthetic plastic causes serious environmental problems due to their non degradability. The intrinsic qualities of durability and resistance to degradation, over the last two decades, they have been increasingly regarded as a source of environmental and waste management problem emanating from plastic materials. Due to their increased usage and threats they pose to environment, need for eco friendly alternative arises. One option is to produce truly biodegradable polymer, a fully biodegradable polymer is defined as a polymer that is completely converted by living organisms, usually microorganisms, to carbon dioxide, water and humus material. These Biodegradable materials include polylactides, polyglycolic acids, polyhydroxyalkanoates (PHAs), aliphatic polyesters, polysaccharides and their co-polymer sand/or blends (Steinbuchel and Schegel, 1991).

Polyhydroxyalkanoate (PHA) is discovered as most suitable alternative of synthetic plastic with many potential biomedical applications, including medical implants and drug delivery. Poly-β-hydroxy butyrate, is the well known member of the PHA series of polyesters, accumulates in many bacterial species as a carbon or energy storage material similar to sugar. PHB is a commonly found substance and readily biodegradable aerobically and anaerobically. Lafferty *et al.* (1988) stated that the accumulation of PHA by microorganisms can best stimulate under unbalanced growth conditions, *i.e.*, when nutrients such as nitrogen, phosphorus or sulfate become limiting, when oxygen concentration is low or when the C: N ratio of the feed substrate is higher. PHB is accumulated by numerous microorganisms and is the best characterized PHA (Madison and Huisman, 1999). PHB exists in the cytoplasmic fluid in the form of crystalline granule about 0.5 μm diameter. β-hydroxybutyrate is connected by ester linkage and form PHB. Microbial biosynthesis of PHB starts with the condensation of two molecules of acetyl CoA to give acetoacetyl-CoA which is subsequently reduced to hydroxybutyryl-CoA. This can be extracted from the cells as native granule or by solvent extraction and processed in the same way as polypropylene (Hanzlikova *et al.,* 1985). This latter compound is then used as a monomer to polymerize PHB. The higher production costs, especially raw material costs, make it difficult for PHA plastics to compete with conventional petroleum-based plastics in the commercial market place. Hence, alternative strategies for PHA production are being investigated. PHA production

costs could be reduced by several means by using cheaper substrates such as starch, whey (Kim *et al.,* 1994) soyabean meal, jaggery and other agro industrial based substrate or enhancement of product yield example, by using recombinant *E. coli* (Lee, 1996). Li *et al.,* (2013) isolated *Pseudomonas mandelii* which synthesized 22.3 g/L of PHB when cultured with sucrose as the sole carbon source. In this present work of investigation, high PHB producing strains will be isolated and identification of gene responsible for PHB production will be carried out, which can be further used for gene cloning and bacterial transformation for increased PHB production in future studies. This piece of work also include creation of mutant strains for enhanced PHB production followed by comparative analysis of wild type and high PHB producing mutant strain with respect to PHB producing genes. This study will be carried out with the following objectives:

Aims and Objectives:

- Isolation, screening and molecular characterization of PHB producing bacterial isolates.
- Optimization of media for increase in PHB production.
- Chemical characterization of recovered PHB by determining melting temperature, structural characterization by FTIR, NMR and mass Spectroscopy.
- Identification of PHB producing genes and development of mutant bacterial strains for increased PHB production.
- Comparative analysis between wild type and mutant with respect to PHB producing genes.

Review and Literature:

Among the most investigated biodegradable polymers, Polyhydroxyalkanoates are superior to other biodegradable polymers because of the large number of different monomer constituents that can be incorporated. At present, about 150 different hydroxyl alkanoate units have been identified. The most common is poly-β-hydroxybutyrate (PHB). The PHB is non-toxic, biocompatible and biodegradable thermoplastics that can be produced from renewable resources. They have a high degree of polymerization; are highly crystalline, optically active and insoluble in water. These features make them highly competitive with polypropylene, the petro chemically derived plastics.

The first and the best known member of PHA, the homo polymer poly-β-hydroxybutyrate (PHB) were discovered by Maurice Lemoigne in 1925 (Jackson and Srienc, 1994). Approximately 150 different hydroxyalkanoic acids are at present known, as constituents of bacterial storage polyesters. PHAs, synthesized by many Gram positive and Gram negative bacteria as storage compounds, are deposited as insoluble inclusions in the cytoplasm (Steinbuchel and Valentin, 1995). Plastics produced from PHAs have been reported to be truly, fully biodegradable. Microbes belonging to more than 90 genera including aerobes, anaerobes, photosynthetic bacteria, archaebacteria and lower eukaryotes are able to accumulate and catabolise these polyesters. PHB is an aliphatic homopolymer of poly-β- hydroxybutyric acid with a melting point of 179 °C and is highly crystalline (80%). It can be degraded at the temperature above its melting point. PHB has some properties similar to polypropylene with three unique features: thermoplastic processability, 100% resistance to water, and 100% biodegradability (Page, 1995).

PHAs are classified as scl–PHAs, mcl–PHAs and lcl –PHAs based on the length of the hydroxyl alkanoic acid monomers (Steinbuchel and Pieper, 1992). The scl –PHAs are composed of C3 to C5, 3-hydroxy/4-hydroxy fatty acids. Poly-β-hydroxybutyrate (PHB), the first of the PHAs studied extensively, falls in this group of PHAs. mcl – PHAs are composed of C6 to C16, 3-hydroxy fatty acids.

PHB granules act as energy reserve materials when nutrients such as nitrogen and phosphorous sources are available in limiting concentrations in the presence of excess carbon source. Senior and Dawes (1971) proposed that PHAs could serve as a sink for reducing power and, therefore, be regarded as a redox regulator within the cell. Encystment of *Azotobacter* cells in lab conditions was linked to PHB accumulation (Stevenson and Socolofsky, 1973). However, later it was shown that in natural habitats, accumulation of large amounts of PHB was not an absolute requirement for encystment.

Anderson and Dawes (1990) observed that the presence of PHB in a cell retarded the degradation of cellular components such as RNA and proteins during nutrient starvation frequently, but not universally. PHB enhanced survival of some but not all the bacteria and also served as a carbon and energy source for spore formation in *Bacillus* sp. Steinbuchel and Schegel (1991) reported that PHAs were stored as intracellular cytoplasmic inclusions. And, these inclusions, as a reduced polymer, provided the advantage that they become unavailable as a carbon source for the competing organisms, and that they were osmotically inert and did not, therefore, affect the osmotic pressure of the cells. He further added that the PHA biosynthesis in *Ralstonia eutropha* and *Rhodospirillum rubrum* included pyridine nucleotide dependent reduction of acetoacetyl-CoA. Thus, PHB is a sink for reducing equivalents and can be considered to be a fermentation product.

By contrast, in *Pseudomonas oleovorans* and *P. aeruginos*a, oxygen deficiency exerted a negative effect on the accumulation of PHA. Because in these organisms either reducing equivalents were formed or energy in the form of ATP was consumed. In *Rhizobium* and *Bradyrhizobium japonicu*m, during the bacteroid stage, the nitrogen fixation apparatus competed with PHB formation for reducing equivalents (Povolo *et al.,* 1994). Production of PHB increase by creating nutrient deficient condition and by using cheap substrate like corn syrup liquor, whey, soyabean meal, nut oil cake and jaggery etc (Chaijamrus and Udpuay, 2008). Senthil kumar and Prabhakaran (2006) evaluated different bioeffluents such as Molasses, Sago and Thippi for PHB production by *Alcaligenes eutrophus* MTCC 1285.

PHA recovery processes, in addition to the costs of maintaining pure cultures and the high costs of organic substrates Doi (1990) described a chloroform extraction method for

extraction of PHB which reduce the cost of PHB extraction. Ramsay *et al.,* (1995) examined the recovery of PHA from three different chlorinated solvents (chloroform, methylene chloride, and 1,2-dichloroethane). They obtained the best recovery and purity when biomass was pretreated with acetone. Hahn *et al.,* (1994) used dispersion with sodium hypochlorite and chloroform method for PHB extraction. They claimed that this method removed most of the non-PHA cellular materials during sodium hypochlorite digestion, which facilitated the separation of PHA from the cells.

Thin layer chromatography was used to determine the presence of PHB in production media. Black colour bands were observed in TLC which indicated the presence of PHB in the production medium (Sharmila *et al.,* 2011). PHB produced was characterized with Shimadzu X-RD 6000X, FTIR spectrometer, and 500MHz JEOL ECA 500 H-NMR and C-NMR spectrometer to determine the structure of PHB, FTIR spectrum of PHB revealed characteristic peaks at 1288.4 cm^{-1} and 1728.1 cm^{-1} which correspond to the C–O and C=O stretch respectively. The data was in agreement with XRD, confirming the presence of PHB. The 1H-NMR spectrum of PHB showed a number of characteristic PHB peaks at δ = 5.2, 2.5, and 1.2 which correspond to a —CH doublet, —CH2 multiplet, and –CH3 doublet respectively (De Rooy *et al.,* 2007).

Umeda *et al.,* (1998) reported that in *Acinetobacter sp., Alcaligenes latus, Pseudomonas acidophila,* and *Ralstonia eutropha,* the phbCAB genes are in tandem on the chromosome although not necessarily in the same order. In *Paracoccus denitrificans, Rhizobium meliloti* and *Zooglea ramigera*, the *phb*AB and *phb*C loci are unlinked. The PHA polymerase has two subunits in *Chromatium vinosum, Thiocystis violacea*, and *Synechocystis* encoded by *phb*E and *phb*C genes (Hein, *et al.,* 1998). The PHB biosynthetic genes *phbA* (for 3- ketothiolase), *phbB* (NADPH-dependentacetoacetyl-CoA reductase), *phbC* (PHB synthase) from acetyl-CoA are clustered and located in one operon *phb*CAB. The loci encoding the genes for PHA formation have been characterized from 18 different species (Madison and Huisman, 1999). The diversity of PHB biosynthetic pathways implies how far the *pha*l oci have diverged. The *phb* (genes encoding enzymes for scl-PHA) and *pha* (genes encoding enzymes for mcl-PHA) are not clustered and the gene organization varied from species to species. Six genes encoding proteins related to PHB metabolism are clustered in 2 separate loci, *phb*ZCPR and *phb*AB, in a PHB-

producing bacterium *R. sphaeroides* FJ1 isolated from wastewater. PHB synthase, the key enzyme for PHB synthesis, is constitutively expressed, and its expression level is not affected by different growth conditions (Yang *et al.,* 2006).

Biodegradation is dependent on a number of factors such as microbial activity of the environment and the exposed surface area, moisture, temperature and pH (Boopathy, 2000). Kim *et al.,* (1997) observed that fungal biomass in soils generally exceeded the bacterial biomass and thus it is likely that fungi might play a considerable role in degrading polyesters, just as they predominantly perform the decomposition of organic matter in the soil ecosystem.

Mutation in genes of interest plays important role increasing in PHB production. Katircioglu *et al.,* (2003) determined the putative mutant strains of *Bacillus megaterium* Y6, *B. subtilis* K8, *B. sphaericus* X3 and *B. firmus* G2 were studied for their poly-β-hydroxybutyrate (PHB) production capacities. Mutations were induced by using UV light, acriflavin and 5-bromourasil. For each strain, percentage yield of PHB according to cell dry weight was determined in a range of 1.46-63.45%. Acriflavin-induced mutation resulted in one variant (173A2) which produced 200µg/ml PHB after 24 h of incubation using the modified culture medium with glycerol 1.5% as carbon source (Elsayed *et al.,* 2013).

Material and Methods:

- **Isolation of PHB producing bacterial isolates and screening of their PHB producing ability**

  - **Sampling:** Samples will be collected from the polluted or highly contaminated sites like municipal waste soil, grass rhizospheric soil, composting site, household waste water, sewage sample and salty water etc. of Shekhawati region in Rajasthan.

  - **Isolation:** The collected samples will be subjected to serial dilutions the appropriate dilutions will be plated on nutrient agar medium by spread plate method. The isolated bacterial colonies will be restreaked on Nutrient agar

medium. The pure culture will be stored as slant containing glycerol and will be stored at 4°C.

- **Screening:** PHB producing bacteria will be grown on nutrient agar and diagnosed for presence of PHB granules by using the Sudan black B (Burdon, 1946) and carbol fuchsin staining (Aneja, 2001). PHB granules as inclusion bodies can be visualized after the staining methods.

➢ **Characterization of PHB producing bacterial strains**

- **Morphological Characterization:** Different isolates will be characterized on the basis of morphological characters like colony shape, colony margin, elevation of colony and pigment production of colony on nutrient agar plates etc (Aneja, 2001).

- **Biochemical Characterization:** Bacterial isolates will be identified on the basis of different biochemical tests like Gram's staining, Spore staining, Urea test, Nitrate test, MR-VP, Sugar fermentation test (Aneja, 2001), Motility test (Hunter and Crecelius, 1938), Catalase test (Graham and Parker, 1964), Gelatinase test (Goyal *et al.,* 2012), Oxidase test (kovaks, 1956) etc.

- **Molecular Characterization:**

  Isolation of Genomic DNA: The overnight grown bacterial culture will be used for extraction of bacterial DNA by the protocol as described by Azadeh and Meon, 2009.

  16S rDNA amplification: The isolated DNA will be subjected to 16S rDNA gene amplification with the help of universal bacterial primers for amplification of 16S rDNA gene (Srilakshmi and Ramachandra, 2012).

  Sequencing: Sequencing of amplified DNA will be done by commercial sources.

Phylogenetic analysis: The sequence will be taxonomically identified according to 16S rDNA and compared to sequences available in the NCBI using BLAST to identify the bacterial species involved (Altschul *et al.,* 1990).

- **Optimization of media for increase in PHB production**

To optimize the production media for increasing the productivity of PHB, types of carbon sources (Dextrose, Sucrose, Starch, Mannitol and Glycerol), types of nitrogen source (Tryptone, Peptone, Ammonium Sulphate, Cystein and Soyabean meal), carbon-to-nitrogen ratio of production media and use of mix microbial culture will be investigated (Patel *et al.,* 2012).

- **Extraction of PHB**

PHB will be extracted by solvent dispersion method using sodium hypochloride and chloroform (Dhingra and Kumari, 2013).

- **Preliminary identification of PHB**

The formation of crotonic acid in the presence of concentrated sulfuric acid identifies the production of microbial PHB (Singh, 2011). Thin layer chromatography will be used for preliminary identification of PHB (Srilakshmi and Ramachandra, 2012).

- **Characterization of recovered PHB**

Melting temperature will be estimated with the help of "Programmable melting point apparatus by Veego" (Chaijamrus and Udpuay, 2008). Presence of functional group will be identified by FTIR, the protonic vibration in PHB molecule will be determined by NMR spectroscopy. Molecular weight will be determined using Mass spectroscopy (Ailing *et al.,* 2010). NMR and Mass spectroscopy will be accomplished through the commercial sources.

- **Identification of PHB producing genes**

The genes that are considered to be essential in the PHB biosynthetic pathway, phaB (acetoacetyl coenzyme A reductase) and phaC (PHA synthase), will be identified in

bacterial strain and sequenced. Gene responsible for PHB production will be amplified by PCR using literature based specific primers and identified subsequently by gene sequencing (Galehdari *et al.*, 2009).

- **Development of mutant strains for increasing PHB production**

  Strain improvement will be carried out by physical mutagen like ultraviolet irradiation and chemical mutagen such as ethyl methanesulphonate (EMS). The best mutant with respect to PHB production use will be found by trial and error. (Sreeju *et al.*, 2011).

- **Comparative analysis between wild type and mutant with respect to PHB producing genes**

  Comparative study will be made between wild type and effective mutant to find out the genomic difference between them with respect to PHB producing genes by using the multiple sequence alignment tools of bioinformatics like CLUSTALW (Aneja and Charles, 1999).

- **Publication of research results and thesis writing**

  The Publication of research results in National/ International journals and conferences would be carried out.

Findings:

In the 21st century we are living in huge load of pollutions from many sources including polythene wastes. Plastics being xenobiotic are recalcitrant to microbial degradation. Plastic production also involves a number of harmful chemicals which pose environmental as well as human health risks. Nature's built-in mechanisms and self-regulation ability cannot tackle novel pollutants since these are unfamiliar to it. In the recent years, there has been increasing public concern over the harmful effects of petrochemical-derived plastic materials in the environment. There is an obvious need to minimize the generation of plastic waste and to develop technologies that can play a vital role in mitigating these problems.

Hence the search for a suitable, economical, harmless alternative is of huge demand. Bioplastics are the most suitable for this cause. Poly-β-hydroxybutyrate is naturally-occurring polymers produced by bacteria. These bioplastics are being used in various applications such as coating, laminations and biodegradable products. It has gained major importance due to their structural diversity and close analogy to synthetic plastics and these are gaining more and more importance world over. PHBs have functional properties sufficient to replace a significant portion of the 300 billion pounds of petroleum-based plastics used worldwide today. It is biodegradable, biocompatible and produced by microorganisms by utilizing renewable source of carbon. However, much more effort is required in this area to increase the production of inexpensive bioplastic for the successful replacement of the non-degradable plastics. Thus the future of bioplastic depends on the efforts towards fulfilling requirements of price and performance. Research efforts have specifically addressed the issue of productivity. Root nodules, soil and water resources are rich in various nutrients as well as they provide many environmental stress conditions to their inhabitants which are the ultimate resources for PHB producers. In this regard, the current study revealed the presence of many PHB producers in both the environments studied which can be used for production of bioplastics in both laboratory as well as industrial scale. Hence in the present study, non-discriminate isolation of bacteria from various sources such as root nodules, rhizospheric soil, composting soil, municipal waste soil, sewage water and salt water were carried out in order to obtain PHB producers. Sudan Black B and carbol fuschin stains were used for the identification of PHB granules

inside the cell. Based on the intensity of stains used to check PHB production, nine isolates were selected. Identification of these isolates was carried out according to morphological, biochemical and molecular techniques. From these tests isolates found to belong *Bacillus sp., Bacillus megaterium, Bacillus thuringiensis, Bacillus tequilensis, Bacillus subtilis, Pseudochrobactrum asaccharlyticum, Paenibacillus sp.* and *Alcaligenes faecalis.*

The product formation is strongly influenced by the media components and thus the optimization of media components plays an important role in any fermentation process. The incubation time of 72h was found to be best for PHB production by all the selected isolates. Maintaining the pH and temperature of the medium at 7.0 and 30°C was found optimum for PHB production by all the selected nine isolates. These nine isolates were tested for their ability to utilize a wide range of diverse carbon substrates, Glycerol was found to be the choice of substrate for PHB production by all isolates. Ammonium sulphate supplementation was found to be best nitrogen source by all the isolates. Maintaining the C:N ratio of 16:1 using the best C and N source was found to be optimum.

Out of nine natural promising isolates M.Com2(1) and Sw/Tw(3)were found to be the most efficient PHB producers. Under optimized conditions, M.Com2(1) produced a PHB yield of 24.51%(w/w) while Sw/Tw(3) produced 19.81%(w/w). M.Com2(1) performed better than the reference strain *C. nector* in PHB production. These two bacterial isolates were checked for PHB production by using as mixed inoculums. Mixed inoculums were produced 22.06% (w/w) PHB when M.Com2(1) and Sw/Tw(3) were present in the ratio of 3:1.

The biopolymer was extracted from the bacterial pellet using dispersion of sodium hypochlorite and chloroform solution. The primary detection of extracted polymer was made by crotonic acid formation and thin layer chromatography. The extracted PHB was characterized by DSC, FTIR, ^{1}H NMR and GCMS confirmed as PHB. The characterization of PHB by various analytical techniques showed the production of pure PHB by the selected isolates. From differential scanning calorimetry melting temperature of polymer was analyzed. The melting temperature was 176°C. FTIR is one of the powerful and rapid tools to obtain information on polymer structure. The spectrum

indicated a transmittance band at 3430cm^{-1}, 2976cm^{-1} and 1724 cm^{-1} which represents the presence of terminal OH group, methylene C-H vibrations and carbonyl group respectively.

Nuclear Magnetic Spectroscopy (NMR) is a powerful technique used for elucidating the chemical structure of the compounds. The ^{1}H NMR spectrum of PHB showed three characteristic groups signals such as a doublet at 1.30 ppm which is characteristic of methyl group, a doublet of quadruplet at 2.52 ppm which is attributed to methylene group and a multiplet at 5.29 ppm characteristic of methylene group. The esters and their derivatives obtained after reaction of sample showed fragmentation patterns in GCMS that enabled to define the structure of PHB obtain from *Bacillus megaterium* MS5 (M.com2(1)).

Attempts were made to improve the *Bacillus megaterium* MS5 (M.Com2(1)) for the enhanced production of PHB. The improvement of microbial strains for the over-production of industrial products has been the hallmark of all commercial fermentation processes. Such improved strains can reduce the cost of the processes with increased productivity and may also possess some specialized desirable characteristics. Strain improvement is usually done by mutating the microorganism by classical mutagenesis, which involves exposing the microbe to physical mutagens such as UV rays and chemical mutagens such as EMS. The UV treatment could not generate hyper producing mutants. The chemical mutant such as EMS was checked for their efficiency to generate hyper producing mutants and EMS treatment resulted in eleven mutants. Out of these eleven, four showed increased biomass and PHB production than the wild type strain.

The biodegradability made the biopolymers useful the in the usage for replacement of synthetic plastic to reduce garbage accumulation and controlled release and drug targeting of pharmacologically active substances. Hence the biodegradability was checked with biological and sterile distilled water. Biological medium was found to more suitable medium for PHB degradation.

The pha operon of *Bacillus* consist of five genes such as *phaP, phaQ, phaR phaB* and *phaC*, where the *phaRBC* genes are in one orientation under the control of a single promoter; *phaP* and *phaQ* are in separate orientation. In Bacillus the gene coding for

βketothiolase (*phaA*) is not associated with pha operon. In the present study PHB biosynthesis genes were identified and sequenced. PCR identification strategy was used to isolate the genes from *Bacillus megaterium* MS5 (M.Com2(1)). The primers were designed to amplify PHB biosynthesis genes such as *phaB* and *phaC*. All the genes characterized were of complete ORFs of expected size with a start site and a termination codon. The gene sequences were used to study the gene polymorphism and sequence homology. The *phaB* gene *Bacillus megaterium* MS5 and its mutant was 744 bp long. The sequence showed similarity with acetoacetyl CoA reductases of various *Bacillus spp*. The PHA synthase of *Bacillus* is reported as a unique one, which requires a *phaR* protein sub-unit for its activity. The gene coding for synthase was also identified and characterized from *Bacillus megaterium* MS5 and its mutant strain. The gene was 1089 bp long complete ORF and was similar to that of *B. megaterium*. To observe the nucleotide differences in wild type and mutant strains gene sequence ClustalW was used. Single nucleotide changes were identified in both the mutated *phaB* and *phaC* gene sequences as compare to wild type *phaB* and *phaC* gene sequences. Nucleotide base G is replaced by T in mutant it shows the effect of EMS. Nevertheless, there are several problems that remain to be solved and could be improved on and even tailored to meet various needs. The search for the favourable strains of PHB producers is an endless process and improvement of resourceful poly-β-hydroxybutyrate producing bacteria has become the need of the hour and engenders copious scopes for research in this field.

Significance of Work:

- The foremost significance of this work lies in isolating novel bacterial strain or creating a mutant strain for enhanced PHB production at industrial level.
- The high cost of PHB production and extraction is main problem associated with the use of PHB for making biodegradable plastic in market. This study will be able to evaluate the appropriate carbon and nitrogen sources for PHB production and the feasibility of maximizing PHB production by optimizing growth conditions such as temperature and pH which would further decrease the production cost.
- Increased production of bioplastics can significantly reduce carbon di oxide emission, continuous synthetic plastic waste generation and decrease the consumption of petroleum based plastic.
- Other than these outcomes, this project is also important for Rajasthan because from August 2010, the Rajasthan government banned the use of plastic bags across the state due to adverse effects of plastic in environment. Hence, this work would be very useful for Rajasthan and other states where synthetic plastic is banned.

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